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14. ABSTRACT Recombinant human lubricin (rhPRG4) was expressed by transfected CHO-S cells at a level of secretion that may be scalable and thus enable production of a GMP protein for clinical use. A purification bioprocess was created that achieved a purity level of approximately 95%. A total of 5 bands visualized on SDS-PAGE are all product related following excision and analysis by LC-MS. Full-length and truncated rhPRG4 constructs are being tested for chondroprotective ability. The full-length construct significantly lowers friction between pressurized discs of articular cartilage. The coefficient of friction in the presence of rhPRG4 was 0.03 whereas for a saline control 0.07. Study of the 5 other constructs and confirmation of reduction in levels of chondrocyte apoptosis attributable to lower friction is ongoing. The post-translational glycosylations on rhPRG4 appear to be (1-3)GalNac-Gal (2,3) NeuAc judging by sequential enzymatic deglycosylation and molecular weight shift on SDS-PAGE. Western blotting of rhPRG4 with mAb 9G3, which reacts with the glycosylations in the lubricin mucin domain, also confirms that the glycosylations are consistent with lubricin secreted by human synovial fibroblasts. Analytical ultracentrifugation shows that there are 5 distinct species in rhPRG4, 3 of which are major forms: monomer, dimer and a higher order tetramer. This biophysical form of analysis may also prove useful in a quality assurance program in GMP protein production as the relative amounts of each major form can be monitored.					
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Introduction

We have produced recombinant human lubricin which we hypothesize could provide disease modifying relief to patients at risk for post-traumatic OA of the knee. The epidemiologic civilian experience indicates that 18% of the total OA burden is of traumatic origin. Among military servicemen and women, acute knee injuries comprise almost 5% of all reported injuries. Intra-articular (IA) injections of recombinant lubricin in pre-clinical rodent models show a chondroprotective effect upon cartilage structure and chondrocyte metabolism. Lubricin is a mucinous glycoprotein which we show can be manufactured by CHO-S cells. The point of this work is also to establish that the production of this recombinant human protein is scalable, to identify a bioprocess for purification and to ascertain that recombinant protein is active in an in vitro cartilage explant bearing system. Strategies to enhance the production of this mucin-like protein by truncating the mucin domain while still retaining low friction properties is also a goal. Finally, upon identifying the candidate recombinant lubricin that possesses scalability and chondroprotective bioactivity in the form of low friction and caspase-3 activity in the cartilage bearings, this candidate will undergo clonal expansion. Enough lubricin will be purified to enable a trial in swine to determine if IA lubricin is chondroprotective following ACL transection, and in another cohort with ACL reconstruction, in an attempt to minimize cartilage loss. All of the reagents and cell lines used are readily available such that the technology can be transferred to a future turn key protein manufacturer for clinical translation.

AIM 1: Designing and implementing a laboratory grade production of therapeutic lubricin candidates which can ultimately be replicated by a well established turn-key partner for protein production who possess the capability to manufacture a large glycoprotein. **Rationale:** A potentially therapeutic lubricin must be identified, structurally defined and its mode of expression and purification determined, which will directly impact its likelihood of manufacture.

Manufacture of Lubricin: Feasibility of Cell-based Manufacture

We have succeeded in expressing all 5 of the shortened lubricin (PRG4) products as well as the full-length (PRG4) product in CHO-S cells from ATCC. The SDS-PAGE in Fig 1 shows the results of expression of each of these. Digestion of the full-length product with NaNase I and O-glycosidase DS also confirms that the post-translational glycosylations are O-linked \square (1-3)GalNAc-Gal side chains (Fig 2). Upon digestion the molecular weight of the core is 151 kDa as predicted for the full-length PRG4 of 1404 amino acids. The yields from cell factories for each of the clones producing the 6 rhPRG4's illustrated exceed 25 ug/ml. We have optimized the culturing conditions in cell factories and determined that based on yield alone that the full-length rhPRG4 and constructs 2 and 3 are prime candidates for scalability. These results will be rationalized against their friction reducing activity in Aim 2 efforts.

Design of Bioprocess for Purification of Lubricin from Conditioned Culture Media

Transfected CHO-S cells were grown in cell factories to cell densities exceeding 1×10^6 cells/ml as described in the initial grant application. Conditioned media was decanted and clarified by centrifugation (100 mL) and diluted with 5 mL 200 mM Tris, 40 mM $MgCl_2$, pH 8.2 and mixed with 40 units of Benzonase (250 units/ μ l, Novagen) to remove soluble polynucleotides. The solution was mixed for 2 hours at room temperature, then mixed with urea to a concentration of 1M, resulting in a 120 mL of solution. To this was added 1N NaOH to adjust to pH 11 and 0.01% Tween 20 (sorbitan monolaurate, Sigma).

The post Benzonase material was next treated using DEAE anion exchange resin with pH of 5.5 in the presence of 1M Urea and 0.01% Tween 20 run in bind and elute mode where the product binds to the resin and the contaminants do not. The column was first sanitized with 0.1N NaOH on an AKTA Purification System (GE Medical) which was acquired during effort in Year 1; then

charged with 100mM NaHPO₄, 1.5M NaCl, pH 7.2; and re-equilibrated with 200mM Tris-HCl, 1M Urea, pH 5.5. The 30 ml volume column was then loaded with the 120 ml solution at 2 ml /ml resin at a flow rate of 5 ml/min (240cm/hr), followed by a wash with equilibration buffer - 100mM Tris-Borate, 100mM NaCl, 1M Urea, 0.01% Tween 20, pH 11. Shortly after loading, product was collected through a step wise addition of 1M NaCl (290mL total volume). The high concentration NaCl was removed via buffer exchange using a 100 kDa molecular weight cut-off Pellicon flat sheet membrane Ultra Filtration Diafiltration filter (UFDF)

(Millipore). The diafiltration buffer was 25 mM NaPO₄, 150mM NaCl, pH 7.2 (PBS). After sanitization with 0.1N NaOH; a rinsing with MilliQ water; and equilibration with 25 mM NaPO₄, 150mM NaCl, pH 7.2, the membrane was operated with a cross-flow ~120 ml/min ; transmembrane pressure = 6-10 psi; permeate flow = ~15-20 ml/min to concentrate the solution to approximately 12.5 ml.

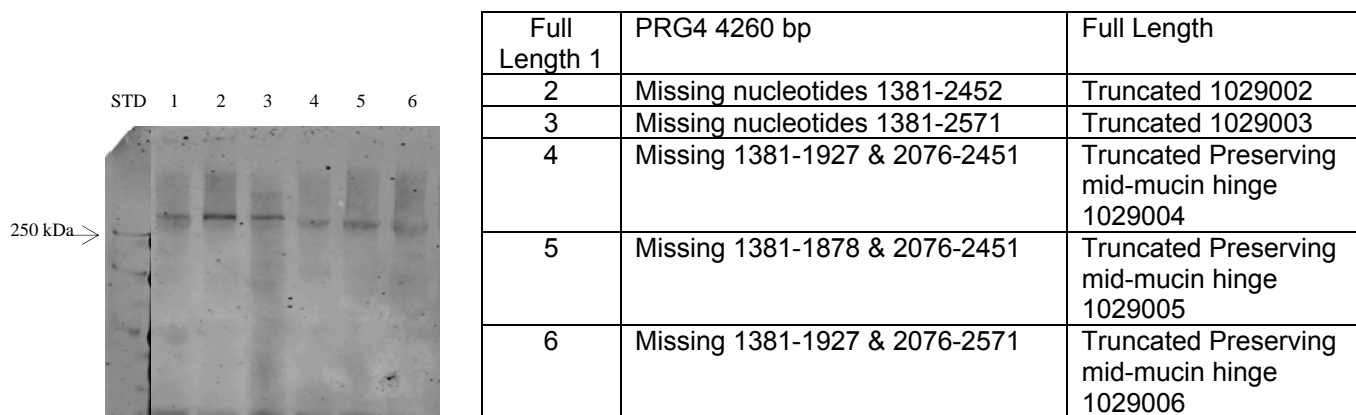
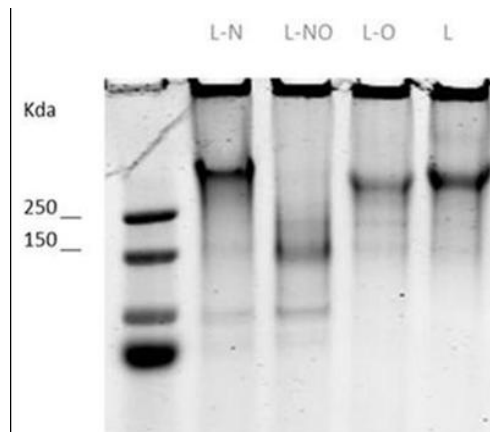


Fig 1. Western blot of full-length lubricin and 5 truncated versions probed with mAb 9G3 which recognizes the glycoproteo epitopes of the lubricin mucin domain. Note that the apparent molecular weights decrease in order with the expected molecular weight from successive truncation of the rhPRG4.

Fig 2. Digestion of rhPRG4 Construct #1 with Neuraminidase (NaNase 1) and O-glycosidase DS exposes the molecular weight of the amino acid core of rhPRG4 as revealed in the lane labeled L-NO. The predicted molecular weight of the core is 151 kDa which is experimentally confirmed by this SDS-PAGE. Digestion with Neuraminidase or Glycosidase DS alone has little effect on molecular weight as digestion with O-glycosidase DS which removes O-linked $\beta(1-3)$ GalNAc-Gal residues likely needs to have the terminal Neuraminic acid removed. This confirms that the protein is roughly 50% w/w glycosylated and that the $\beta(1-3)$ GalNAc-Gal O-links are capped in many instances but not all with neuraminic acid.



This partly purified bind and elute lubricin pool was pH adjusted with 50 mM pH = 7.5, and passed through a hydroxyapatite column (BioRad CHT), Column Volume – 5ml, Column Load – 20 ml Load/ml resin, Flow rate = 5 ml/min. The column was first sanitized with 0.1N NaOH and 1 M NaCl, charged with 500mM NaHPO₄, pH 6.5; equilibrated with 500mM NaHPO₄/1M Urea,

pH 7.4. The bind and elute pool was eluted with 15mM NaHPO₄, 1M Urea, 0.01% Tween 20, 1M NaCl, pH 7.4, to produce 70 ml of bind and elute containing the product.

To concentrate and buffer exchange, the post HA exchange flow-through product pool was filtered using a 100 kDa molecular weight cut-off Pellicon flat sheet membrane Ultra Filtration Diafiltration filter (UFDF) (Millipore). The diafiltration buffer was 25 mM NaPO₄, 150mM NaCl, pH 7.2 (PBS). After sanitization with 0.1N NaOH; a rinsed with MilliQ water; and equilibration

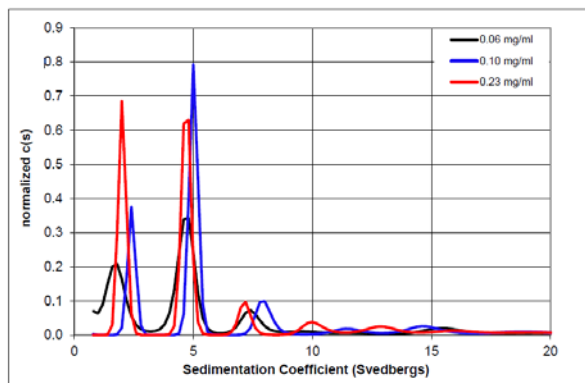


Fig 3. Analytical ultracentrifugation experiment reveals 5 distinct species within purified rhPRG4 Construct #1. Three major species are noted which are likely rhPRG4 monomer, dimer and a higher order tetramer. This assay is conducted in the native PBS solvent which the rhPRG4 elutes in and is thus a suitable QC assay to monitor the inherent aggregation which occurs in rhPRG4 and is a component of lubricin's mucin-like surface protecting bioactivity.

with 25 mM NaPO₄, 150mM NaCl, pH 7.2, the membrane was operated with a cross-flow ~120 ml/min ; transmembrane pressure = 8-12 psi; permeate flow = ~15-20 ml/min to concentrate the solution to approximately 125 ml. Lastly, the post UFDF product pool was subject to 0.2 um filtration to remove any bacterial contaminants. The rhPRG4 tested negative for pyrogens using the Limulus amoebocyte lysate test (Cape Cod Associates) and was also tested for rodent virions by Charles River Laboratories which also tested negative.

Recombinant PRG4 is polydisperse due to its origins as a bio-similar mucin or mucinous glycoprotein. We have observed the anticipated monomer and dimerized rhPRG4 as well a few lower molecular weight fragments and a very high molecular weight that structurally maybe a dimer of a dimer (tetramer). This is typical of mucins which self-aggregate and in this case possibly forming a tetramer. All 5 of these bands were excised from SDS-PAGE and sequenced and matched to human PRG4 in GenBank. They are all product related. These products were also studied by analytical ultracentrifugation. This approach is an industry standard and is used to rationally understand protein aggregation which in the case of lubricin is critical since it is mucin-like and will spontaneously aggregate which is a product-related feature. The analytical centrifugation performed by the University of Connecticut Analytical Ultracentrifugation Facility showed that rhPRG4 has three major distinct populations: 1.0 < S < 3.5 [20%] & 3.5 < S < 6.0 [35%] & S > 6.0 [45%]. Up to 5 species were found by ultracentrifugation (Fig 3) which supports the SDS-PAGE results of 5 bands being PRG4 related following excision and sequencing by liquid chromatography-mass spectrometry.

AIM 2: Identify a candidate lubricin from Aim 1 which has maximal chondroprotection in an *in vitro* cartilage bearing model, which complements scalability, and a low cost-of-goods. **Rationale:** Identification of a lubricin candidate, which is destined for GMP or GMP-like production, should demonstrate a reproducible *in vitro* ability to reduce COF and promote chondrocyte survival. These experiments are being conducted using osteochondral bovine and porcine discs obtained from the femoral condyle of knees obtained from a local abattoir. The lower and larger 12 mm diameter disc is also decalcified and provides an experimental way to rationally predict which of the expressed 6 PRG4 constructs demonstrate the lowest friction

reduction and caspase-3 activity. Therapeutic candidates from Aim 1 are presently being studied using these two dependent variables relative to their anticipated scalable costs.

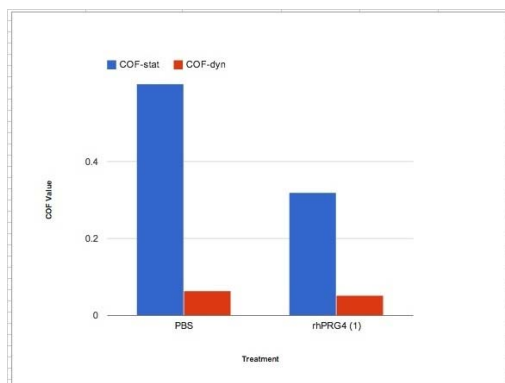


Fig 4. Coefficient of friction (COF) reduction by full-length rhPRG4 in a porcine femoral condyle osteochondral bearing system measuring 6 and 12 mm diameter mounted in the Bose Enduratec. The compressive strain was 18% and remained constant during surface reciprocation. Static COF was significantly lowered by the rhPRG4 compared to PBS in the cartilage explants that had resident lubricin removed.

Using the purification procedures which were innovated in Aim 1 we have purified the 6 different lubricin species and begun lubrication studies for friction reduction bioactivity. This has also required a retooling of the *in vitro* friction test system which oscillated osteochondral explants as bearings while mounted in an Enduratec (which was acquired as part of year 1 activities). These bearings have resident porcine lubricin on their surfaces which is being removed with a 1 hour hyaluronidase digestion followed by a 30 min chymotrypsin digestion. As expected we have observed a significantly higher degree of friction following removal of superficial zone lubricin from these surfaces when lubricated by phosphate buffered saline (PBS) (Fig 4). This has permitted a more accurate assessment of the chondroprotective activity of rhPRG4. Fig 4 shows the significant effect of static friction reduction attributable to rhPRG4 full-length construct #1. Nominally, the static COF for a porcine or bovine synovial fluid lubricated bearing like this is 0.2. This first indication of the ability of rhPRG4 to lubricate a cartilage bearing is significant although we don't yet have the immunohistochemistry from these experiments to confirm cellular chondroprotection. The remaining 5 rhPRG4 constructs are being tested for friction reduction as of this writing.

Aim 3: (Not Begun). In this study we will utilize a large animal model to evaluate whether intra-articular injection of rhPRG4 following ACLT will preserve cartilage after delayed ACL reconstruction (ACLR) surgery. **Rationale:** Large animal studies are a FDA required prelude to human studies. The porcine diarthrodial joint is biomechanically more similar to human joints than those of other animal models.

Timeline

Activities	FY	12	13	14	15
Production of 5 truncated and full-length recombinant human lubricin in CHO-S cells					
Study the 6 lubricin proteins in a cartilage bearing system to identify the one with the lowest cost and highest activity.					
Randomized and controlled trial of best candidate lubricin in a large animal model of PTOA induced by ACL transection.					

KEY RESEARCH ACCOMPLISHMENTS

- Expression of rhPRG4 from CHO-S cells which is a readily available expression platform
- Successful expression of truncated constructs
- Purification of full-length constructs
- Demonstration that the full-length construct lubricates a cartilage bearing in vitro
- Demonstration that ultra centrifugation can be used to quantify lubricin aggregation which is characteristic of mucin products and will be useful as a production quality control

REPORTABLE OUTCOMES

Publications

1. Das S, Banquy X, Zappone B, Greene G, **Jay G**, Israelachvili J. Synergic interactions between grafted Hyaluronic acid and Lubricin provide enhanced wear protection and lubrication. *Biomacromolecules*. 2013 May 13;14(5):1669-77 [PMID: 23560944]
2. Waller, K., Zhang, L., Elsaid, K., Aslani, K., Warman, M., Fleming, B., **Jay, GD**. The Role of Lubricin and Boundary Lubrication in the Prevention of Chondrocyte Apoptosis. *PNAS*. 2013 Apr 9;110(15):5852-7 [PMID: 23530215]
3. Chang DP, Guilak F, **Jay GD**, Zauscher S. Interaction of Lubricin with Collagen II Surfaces Adsorption, Friction, and Normal Forces. *J. Biomech*. 2013 (accepted).

Presentations

1. **Jay, GD**: Tribosupplementation: The Point and Rationale. International Society of Intraarticular Treatment. Rome, Italy, October 3rd, 2013.

Abstracts

1. Teeple E, Larson K, Zhang L, **Fleming B, Jay GD**. Arthroscopic Irrigation of the Bovine Stifle Joint Increases Cartilage Surface Friction and Superficial Zone Chondrocyte Apoptosis. Orthopedic Research Society. Submitted for ORS 2014.

CONCLUSION

The manufacture of recombinant human lubricin (rhPRG4) appears scalable and can be accomplished using CHO cells as a protein production platform. The rational selection of a truncated rhPRG4 as opposed to a full-length protein is ongoing as more data is collected on chondroprotective ability and expression levels in conditioned media. The GMP production of rhPRG4 will represent the first commercialized mucin-like protein.

APPENDIX

Abstract Submitted to ORS 2014

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Activity: ORS Annual Meeting Abstract Submission

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Arthroscopic Irrigation of the Bovine Stifle Joint Increases Cartilage Surface Friction and Superficial Zone Chondrocyte Apoptosis

Author Block: Erin Teeple, MD¹, Katherine Larson², Ling Zhang, MD³, Braden C. Fleming, PhD⁴, Gregory D. Jay, MD-PhD³.

¹Department of Occupational and Environmental Medicine Harvard School of Public Health, Boston, MA, USA, ²Brown University, Providence, RI, USA, ³Department of Emergency Medicine Brown University Medical School, Providence, RI, USA, ⁴Department of Orthopedic Surgery Brown Medical School/Rhode Island Hospital, Providence, RI, USA.

ABSTRACT

Introduction: Arthroscopic knee surgery is among the most commonly performed orthopedic procedures in the United States (1). While arthroscopic partial meniscectomy addresses acute mechanical symptoms, rates of osteoarthritis (OA) progression following this procedure are high. In one study, 48% of patients had developed radiographic OA at 15 to 22 year follow up (2). The causes of OA progression after this procedure most likely include a combination of direct cartilage damage, altered joint contact mechanics, and inflammatory damage. There are no current treatments known to reduce the risk of degenerative joint disease following arthroscopic partial meniscectomy. One potentially damaging consequence of arthroscopic irrigation that has not been well-studied is the dilution and removal of protective synovial fluid lubricants, specifically the mucinous glycoprotein lubricant lubricin and hyaluronic acid. The contribution of inadequate surface lubrication to progressive cartilage damage following arthroscopic irrigation is unknown, but a recent study by Waller et al. using cultured articular cartilage plugs found that mechanically tested cartilage samples lubricated by solutions lacking lubricin demonstrated a greater percentage of apoptotic cells compared to cartilage samples lubricated with either human synovial fluid or purified human synoviocyte lubricin (3). The purpose of this study was to investigate the effects of arthroscopic irrigation on cartilage surface friction and chondrocyte viability using fresh intact bovine stifle joints. The hypotheses of this study were that (1) arthroscopic joint irrigation would increase articular surface friction, and (2) elevated articular surface friction following arthroscopic irrigation would result in increased chondrocyte apoptosis as measured by expression of the apoptosis marker activated caspase-3.

Methods: Fresh bovine stifle joints with the capsule intact were obtained on the day of slaughter from a local abattoir. For the Control specimens (n=4), a capsulotomy was performed and synovial fluid was collected from the joint for use as a lubricant during mechanical testing. For the Arthroscopy specimens (n=4), arthroscopic irrigation was performed as follows: an arthrotomy was made in the superior capsule and an arthroscopy cannula was introduced. The joint was irrigated with 6L of lactated ringers solution at room temperature at a fluid pressure of 55 mm Hg. The irrigant fluid was collected after 3L had been cycled through the joint and saved for use as a lubricant during mechanical testing. For both Control and Arthroscopy knees, paired, full-thickness osteochondral plug bearings of 6 mm and 12 mm diameter were cored out from the anterior, middle, and posterior load-bearing regions of the medial femoral condyle. Static and dynamic coefficients of friction (COF) were measured using a Bose 3230 - AT Series II material testing system (Bose, Framingham, MA). The plugs were kept moist with test lubricant prior to testing and additional lubricant was applied to the cartilage surfaces prior to testing. During friction testing, a 12 N compressive load was applied across the plug surfaces followed by an 8 minute dwell period to allow for stress relaxation. The large plug was then rotated relative to the small plug for 12 rotations of 720° while torque was recorded. Coefficients of friction were calculated as $COF = \tau / ((2/3) * (r) * (load))$, where r = measured radius of the small plug (3,4). Static COF was calculated from the maximal torque measured during the first 20° of rotation and the equilibrium load. Dynamic COF was calculated using the

equilibrium load and the average torque measured during the last 720° rotation. Immediately following testing, the 12 mm plugs were immersed in formalin for a minimum of 72 hours prior to decalcification and paraffin embedding for histologic analysis. Sections from the central contact area of each plug were stained for activated caspase-3, a marker of apoptosis, and counterstained with DAPI. Chondrocyte apoptosis was quantified as the percent of nuclei costaining for DAPI and red fluorescence divided by the total number of nuclei at the following depths from the cartilage surface: (A) 0-100 µm; (B) 100-200 µm; and (C) 200-300 µm. Three 10X views were used from each section to obtain an average score for the plug unless artifact prevented the visualization of three separate surface areas. Results for static COF, dynamic COF, and mean percent expression of activated caspase were compared between groups using unpaired, two-tailed t-tests for each depth and surface location.

Results: Compared to Control specimens, the articular cartilage surfaces from the medial femoral condyle of the Arthroscopic specimens demonstrated a significantly elevated Static COF in the Middle condylar region ($p=0.021$) (Figure 1A) and significantly elevated Dynamic COF in the Middle ($p=0.007$) and Posterior condylar regions ($p=0.002$) (Figure 1B). Caspase-3 activation was found to be significantly greater in cartilage sections from the superficial zone (Zone A) in the middle medial femoral condyle of Arthroscopy specimens compared to Controls ($p=0.044$), but not in the deeper layers (Figure 2).

Discussion: The results of this pilot study support our hypotheses that arthroscopic irrigation of the bovine stifle joint results in elevated cartilage surface friction and increased chondrocyte apoptosis. These findings are consistent with results found by Waller et al. for cultured articular plugs lubricated by PBS versus synovial fluid or purified human lubricin. Our results suggest that arthroscopic irrigation compromises articular surface lubrication at the time immediately following surgery. Modifying the lubricating environment of the joint may provide an opportunity to reduce OA progression following this common procedure. Of note, a recent study using a mouse meniscal injury model found that OA progression was significantly reduced in animals treated with a viral vector to induce lubricin overexpression. It should also be noted that this study used intact joints from otherwise healthy animals, so synovial fluid and articular surface lubricants were likely at physiologic concentrations prior to irrigation and mechanical testing. In the setting of acute joint injury, lubricin levels have been found to be diminished and synovial fluid lubricating ability reduced. Therefore, the vulnerability of chondrocytes to secondary injury post-procedure may be greater clinically than what has been found in this study.

Significance: This is the first study to investigate the consequences of arthroscopy on cartilage surface lubrication and chondrocyte viability. Further investigation is needed to understand cartilage surface mechanics following arthroscopy at post-surgical time points in a living animal model.

Acknowledgments: Supported by: National Institutes of Health (R01 AR050180; R42 AR057276 and P20 GM104937).

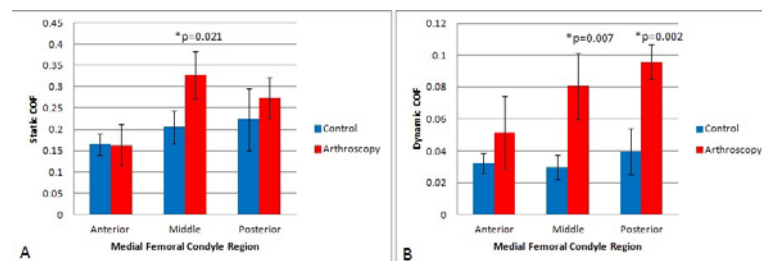


Figure 1: Coefficients of Friction (COF) by region: (A) Static and (B) Dynamic

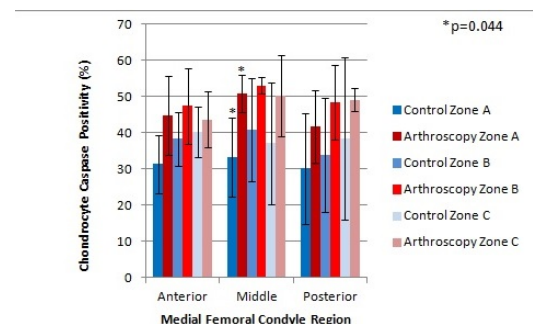


Figure 2: Chondrocyte apoptosis by region and depth zone

References: 1) Kim S et al. J Bone Joint Surg Am 93(11):994-1000(2001); 2)Englund M et al. Arthritis Rheum 50:2811-9(2004); 3)Waller KA et al. Proc Natl Acad Sci USA 110(15):5852-7 (2013); 4) Schmidt TA et al. Osteoarthritis Cartilage 15(1): 35-47(2007).

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Additional Questions (Complete):

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- * Has this abstract or its contents been published in part or in whole?: No
- * Abstract Description: Basic Science
- * Were issues of sex and/or gender differences considered in this research study?: No

If no, please provide an explanation for why it was not considered pertinent or, if it was pertinent, why it was not studied. : This is a pilot study. Also, only male stifle joints were available from the abattoir where the specimens were obtained.

Category for Review (Complete): Cartilage and Synovium - Mechanobiology ; Trauma - Joint Injuries and Post-Traumatic OA

Presentation Type and Disclosures / Agreements (Complete):

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- * 1. Are your research subjects living humans or animals?: No (go to item 2)
- * 2. Were any patient records or imaging studies reviewed for this study?: No (go to item 3)
- * 3. Does this research use only biomechanical testing, data from published articles, databases or specimens that are publicly available?: Yes (IRB approval not needed, go to FDA Status – Skip items 4-8)
- * 4. Human Subject Research: Was this research approved by an Institutional Review Board (IRB) or Ethics Committee? Not Applicable
- * 5. Human Subjects: Number of subjects? Enter 0 if no subjects were used. : 0
- * 6. Type of Investigation: Not Applicable
- * 7. Randomization: Not Applicable
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